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Table of Contents:

	Page Number
<i>Introduction</i>	1
<i>Results/Conclusion</i>	6
<i>Future Studies</i>	9
<i>Bibliography</i>	10

INTRODUCTION

Conclusive evidence implicate the ovarian steroid estrogen as one of the key regulators of normal breast growth and differentiation, and as a mitogen in breast tumors [1, 2]. Its function in tumors has led to the use of tamoxifen, an anti-estrogen, as an essential method of therapeutic intervention.[3] Estrogen receptor (ER) is the major mediator of estrogen action in both tumor and normal breast function but its precise role in the signaling pathways has not been clearly elucidated.

Estrogen Effects on Normal Breast:

At the onset of puberty in women, breast tissue undergoes growth and differentiation in response to the increased levels of circulating estrogens, forming the mature tissue. The non-lactating mature breast contains lobules of branched tubulo-alveolar glands with interlobular stroma and adipose tissue. The glands are lined by a simple cuboidal luminal epithelium with myoepithelial and stem cells located at the basement membrane. Surrounding the glands are stromal fibroblasts and myofibroblasts.

As mentioned above, estrogen is the primary hormone that triggers mammary gland maturation during puberty. It stimulates the proliferation of epithelium that ultimately leads to the development in the adult mammary gland of the complex network of branched ducts terminating in end-buds. Most of the proliferation in response to estrogen stimulation occurs in the duct epithelium [4].

Estrogen's effect on the mammary gland is not exclusively proliferative. It also induces the expression of the progesterone receptor (PR), which allows progesterone, acting through PR, to initiate differentiation of luminal epithelium and local proliferation of the end-buds [5]. Both the differentiative and end-bud proliferative signals of PR ultimately lead to the development of secretory alveoli. Thus, in normal breast growth, ER can be seen having both direct proliferative and indirect differentiative functions.

In the context of the adult non-lactating mammary gland the combined effects of ER and PR display a tightly regulated synchrony with the cyclical hormonal changes of the menstrual cycle. In the late follicular/early luteal stage, when estrogen production peaks, an increased proliferation rate and PR induction are observed in the mammary glands [6]. During the mid-to-late luteal phase increased progesterone levels act on the sensitized glands to initiate differentiation and end-bud development. If fertilization of the oocyte and embryo implantation do not occur, estrogen and progesterone levels drop and the end buds involute [6].

When implantation occurs, the corpus luteum of pregnancy continues to produce both estrogen and progesterone. The combined action of these hormones leads to the development of secretory alveoli - which reach a fully differentiated state soon after parturition. During lactation luminal epithelium becomes columnar with an increased level of endoplasmic reticulum marking its secretory function [7]. The myoepithelial and myofibroblast cells surrounding the alveoli contract to aid milk ejection from the glands.

Mammary gland secretion occurs via a mostly modified apocrine mechanism which involves continuous loss of cytoplasm and membrane [8].

Estrogen Effects in Breast Cancer:

As with most cancers, the development of breast cancer is believed to be a multi-step process. Infiltrating ductal carcinoma accounts for the majority of breast cancer cases (~85%) [9]. The progression of breast cancer is believed to begin with atypical hyperplasia, leading to an intraductal carcinoma or carcinoma in situ, that finally give rise to invasive ductal carcinoma [9]. Most often these cancers arise from the estrogen responsive luminal epithelium. Currently, all breast tumors, in addition to being clinically and pathologically staged, are also assayed for the presence of estrogen and progesterone receptors. Responsiveness to tamoxifen, a widely used adjuvant therapy, is best predicted by the presence of ER/PR in breast tumor tissue [3]. The ER negative/PR negative phenotype is usually associated with worse prognosis and more aggressive and invasive cancers [10].

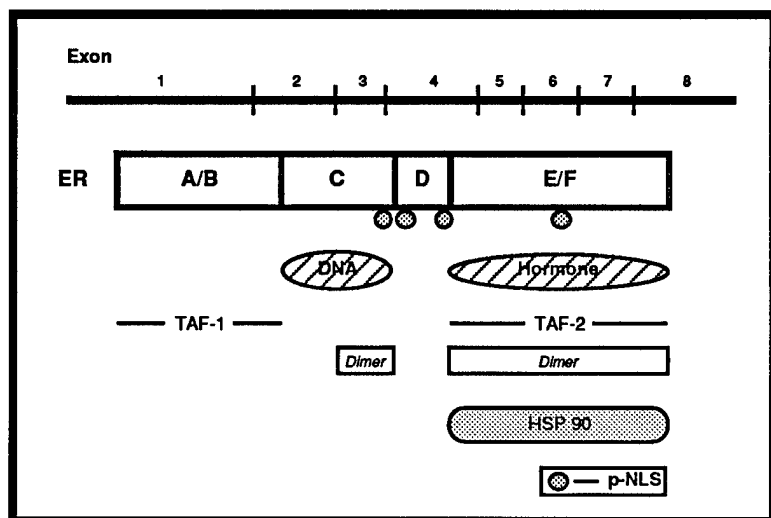
The presence of estrogen receptor as a predictor of responsiveness to tamoxifen therapy is further supported by breast cancer cell line analysis. Such analyses have shown that estrogen's mitogenic signals are mediated via ER, which regulates the expression of several genes involved in proliferation [11, 12]. Two such critical genes products are cyclin D1, a partner of the cyclin dependent kinase 4 (cdk4) involved in the G1-S transition, and myc, a transcription factor essential for cell cycle progression [11, 12]. Both mRNAs and proteins are induced by estrogen, and inhibited by tamoxifen as well as other pure anti-estrogen compounds [11, 12]. The transcriptional control may occur through the classical pathways involving ER interacting with EREs, or alternatively through ER interacting with other transcription factors. In addition, in the case of myc, ER may also be involved in post-transcriptional control of mRNA stability [13].

Another class of estrogen regulated proteins are the proteolytic enzymes: plasminogen activators (PA) (urokinase-PA (u-PA) and tissue type-PA (t-PA), believed to function in the degradation of extracellular matrix by tumor cells during invasion and metastasis [14]. As such, u-PA is an independent predictor of poor prognosis in breast cancer [15]. Although the molecular mechanism of estrogen regulation of PAs remains unclear, induction of proteolytic activity by estrogen implicates ER not only in proliferation but also in the process of tumor invasion.

Consequences of estrogen induction of PR in breast tumors are not as clear. Much of the initial investigation of progesterone action was performed using the endometrium as the model where PR has no effects on proliferation but striking effects on differentiation [16]. The differences between progestin responses of the breast and endometrium do not allow for extrapolation of results from one system to the other. The mixed differentiative and proliferative effects of progesterone in normal breast tissue makes the investigation of its effects in breast tumors complex. Data suggests that in normal cells the interaction of progesterone with its receptor may commit the cells to differentiation, whereas in cells that have been damaged by a carcinogen the proliferative effects of progesterone predominate thus enhancing tumor formation [16].

Estrogen Receptor Structure and Function:

The estrogen receptor (ER) belongs to a family of nuclear steroid receptors that regulate gene transcription. Structural analyses have shown distinct functional domains in the receptor: an amino terminal transactivating domain (A/B), a zinc finger DNA binding domain (C), and a carboxy terminal hormone binding domain (E/F) [17] (Fig 1).



In the absence of hormone, predominantly nuclear ER (four nuclear localization signals), exists in an oligomeric complex with several heat shock proteins (hsps) [18]. When bound to the hsp complex, ER is unable to activate gene transcription [19]. However, in the presence of estrogen, the complex dissociates and the ER dimers are free to interact with palindromic estrogen response elements (ERE's) of genes [20, 21].

Two regions of ER are involved in transcriptional regulation: transcriptional activation function-1 (TAF-1), located in the amino terminus (A/B), and transcriptional activation function-2 (TAF-2) located in the carboxy terminus (E/F) [22, 23]. TAF-1 interacts with other transcription factors independently of estrogen binding. The expression of genes controlled exclusively by TAF-1, such as IGFI and alkaline phosphatase, is not inhibited by the addition of tamoxifen in tissues such as the endometrium [24] [25, 26]. Unlike the hormone independent function of TAF-1, TAF-2 requires estrogen binding for function. The estrogen bound TAF-2, by interacting with TAF-1, induces a class of genes that are sensitive to inhibition by tamoxifen [24]. Although, the two independent TAF's cooperate and enhance each other's transactivation activity, their individual contributions are promoter and cell type specific [18, 22, 23].

Splice Isoforms of the Estrogen Receptor: Function

The estrogen receptor messenger RNA (mRNA) is encoded by a single copy gene in the human genome [27]. Recent studies have determined that multiple forms of the estrogen receptor arise from alternative splicing of the primary ER transcript. Messenger RNAs missing exon 3, 5, or 7 were first identified in primary breast tumors and breast cancer cell lines [28-31]. Initial studies by Fuqua et al. have shown that the skipping of exon 3 (ER Δ 3)

deletes the second zinc finger of the DNA binding domain and renders this form of the receptor incapable of binding DNA [29]. *In vitro* expression of the exon 5 (ERΔ5) and exon 7 (ERΔ7) spliced forms show that both exon skipplings create a frame shift that leads to premature termination of translation, thereby truncating these receptors in different portions of the hormone binding domain. Transient transfection of ERΔ5 in conjunction with an ERE-CAT reporter, has demonstrated constitutive, hormone independent activity of this isoform [30]. My original proposal chose to focus on the function of ERΔ5. At that time studies were ongoing for both the ERΔ5 and ERΔ3 splice isoforms. Subsequently, the data supported the further development of the ERΔ3 isoform project. In addition, we were aware of several other groups who are investigating the role of ERΔ5, using a similar approach as was discussed in my original proposal. Several such groups have recently published their results [32-34]. Accordingly, I will present and discuss data concerning the ERΔ3 splice isoform in breast biology and estrogen responsiveness.

The consequence of exon skipping may have important implications for ER function. The only reported analysis of the distribution of ERΔ3, outside of data in this report, is limited to primary breast tumors and cell lines. Once again only the original characterization performed by Fuqua et al. and limited analysis of breast tumors carried out by Miksicek et al. has identified the presence of ERΔ3 mRNA in breast tumors; however, the problem of tissue heterogeneity complicates the interpretation of these studies [29, 35].

Functional analysis of the ERΔ3 isoform is limited to a series of *in vitro* translation and transient transfection experiments, which demonstrated dominant negative activity of ERΔ3 on ER function [29]. When ERΔ3 was *in vitro* translated with ER, a concentration-dependent inhibition of ER-DNA binding was observed. Inhibition of ER binding to DNA by ERΔ3 was ~25% when a 1:1 ratio of ERΔ3/ER was used and increased to ~90% in a 10:1 co-translation ratio of ERΔ3/ER, thereby suggesting random heterodimerization of ERΔ3 with ER. These data were further confirmed in transient co-transfection experiments of HeLa cells. Transfection of ER and ERΔ3 with an ERE-CAT reporter showed ERΔ3 inhibition of the estradiol (E2)-stimulated ER transactivation [29].

Implications of ERΔ3 on Breast Function:

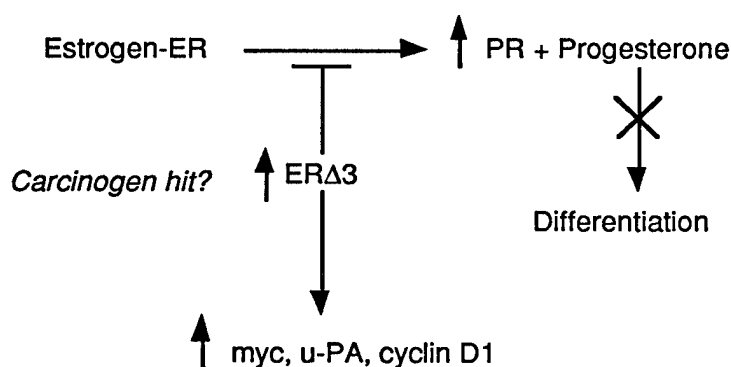
The finding of exon skipped ER isoforms requires re-investigation of estrogen effects in breast biology. We postulate that the existence of ERΔ3 in normal tissue may either present cells with a mechanism for attenuating estrogen responsiveness or, alternatively, potentiate the mitogenic actions of this hormone. Current understanding of the function of this isoform in transcriptional regulation is limited to its inhibition of ER transactivation of a consensus ERE-CAT reporter [29]. If a similar effect can be shown *in vivo*, we can presume that regulation of ERΔ3 expression may present a mechanism for cells to modulate sensitivity to hormone. Alternatively, ERΔ3 may regulate the expression of target genes via DNA binding domain independent interaction with other transcription factors [36].

The involvement of ERΔ3 in breast cancer is still unclear. Tissue heterogeneity of breast tumors complicates the interpretation of the cellular source of ERΔ3 from total tissue. Until specific cellular localization is determined we cannot discriminate between low level of ERΔ3 expression in all cells, versus over-expression in a subtype of normal or tumor cells.

The observation of dominant negative activity of ERΔ3, combined with the existence of alternative pathways involving interactions with other transcription factors, permits us to generate two opposing hypotheses on the possible role of ERΔ3 in breast cancer. Both models involve deregulation of ERΔ3 expression in the process of tumor development. The first model assumes an increase, while the second assumes a decrease of ERΔ3.

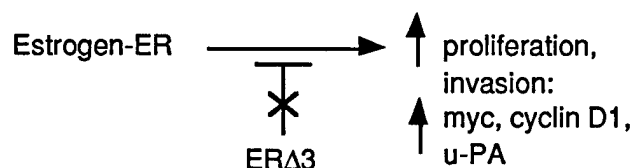
I. In the normal breast ER induces PR in response to estrogen. This induction primes the breast to respond to the differentiative effects of progesterone. In this model, overexpression of ERΔ3 may inhibit estrogen stimulation of PR, thereby potentially allowing escape from the differentiative pathway and forcing cells into the proliferative pathway leading to hyperplasia. In addition, ERΔ3 may, in fact, directly stimulate the expression of genes involved in proliferation by mechanism of regulation not depend on the DNA binding domain (DBD).

MODEL I:



II. The opposing model for ERΔ3 action assumes that a basal expression of ERΔ3 in normal breast is necessary to attenuate the proliferative effects of estrogen. Thus, we can hypothesize that loss of ERΔ3 expression may create hyper responsivity to estrogen stimulation via the classical pathway.

MODEL II:



In order to verify whether ERΔ3 has a role in breast biology and which of the two models more accurately reflects the in vivo situation we are currently performing a series of experiments encompassing analysis of ERΔ3 distribution in normal and tumor mammary tissue, as well as functional effects of ERΔ3 overexpression on growth, invasiveness and gene

regulation. In the next section I will discuss the data from the analyses completed to date and the questions that remain to be addressed.

RESULTS:

Distribution Analyses: ER Δ 3:ER

We screened a series of primary breast tumor tissues for the presence of ER Δ 3 mRNA. In order to facilitate the wide spectrum screen, we employed a semi-quantitative reverse transcription/PCR assay, optimized to reflect both increasing cycle number and total RNA input. Amplified products were size fractionated on replicate agarose gels and transferred onto nylon membranes. For each experiment, filters were probed with gamma-ATP³² end-labeled oligonucleotides. One filter was hybridized with a probe homologous to an internal sequence of exon 4 which recognizes both full length ER and ER Δ 3, while the second filter was hybridized with a probe homologous to a portion of exon 3, which recognize only the full length ER. Both filters were washed under appropriate salt and temperature conditions, and exposed to a phosphorimager screen. Experiments were quantitated using the phosphorimager program ImageQuant to assess the ratio of ER:ER Δ 3 mRNA.

We have observed a range of ratios of ER Δ 3:ER among the breast tumors ranging between 1:2-1:30. With most specimens containing predominantly ER mRNA. The heterogeneity of total ER values and the ER Δ 3:ER ratios is not surprising in view of the well documented cellular heterogeneity of primary breast tumor tissue, with respect to the proportion of normal and tumor cells, and the cellular density of epithelial tumor and intervening stroma [9].

Before the *in vivo* role of ER Δ 3 can be elucidated, a cellular analysis must be performed to clearly identify the source of ER Δ 3 in these tumor tissues, as compared to the content of this isoform in normal breast. The major site of ER expression in the normal mammary gland are the luminal cells. We have assayed several normal cell specimens: three samples of normal myoepithelial mammary cells, four specimens of primary basal/stem epithelial cells, and two samples of luminal epithelial cells (all isolated from reduction mammaplasty tissue and identified using cell type specific keratin markers). In addition we have assayed an immortalized normal myoepithelial cell line (Hs578Bst), and an immortalized normal basal/stem cell line (MCF-10). As previously reported, the MCF-10 cells were ER-. However, in both the myoepithelial primary cells and Hs578Bst cells, we found a ~2:1 ratio of ER Δ 3:ER. Interestingly, the primary luminal cells had a predominance of ER mRNA with a ratio of ER Δ 3:ER of ~1:2. Although preliminary, these results suggest that certain normal mammary epithelial cells contain a ratio that is strikingly different from that seen in breast tumors above, suggesting that ER Δ 3 expression and ER Δ 3:ER ratio varies as a function of cell origin. However, the primary cell purification is especially difficult for luminal cells and we are in the process of collecting more specimens to confirm this finding. This analysis agrees with our assumption that the widely different ratios of ER Δ 3:ER in the breast tumors may reflect the normal/tumor cell heterogeneity of this tissue.

All of the analyses described so far have examined the mRNA levels of ER Δ 3. The protein product of this ER splice isoform has yet to be identified *in vivo*, an analysis complicated by

the lack of antibodies that specifically recognize the protein encoded by this splice isoform, as well as by the low abundance of ER and consequently lower levels of ER Δ 3. A recent report of detection of ER Δ 5 protein in a breast tumor cell line that was previously found to have high levels of the corresponding mRNA species, implies that alternative splicing of pre-mRNA may regulate the expression of ER protein isoforms *in vivo* [37]. Furthermore, in several published studies in which ER has been detected by western blot analysis, less abundant immunoreactive products of lower molecular weight than full length ER were noticed. In at least two such blots, a band is seen approximately in the size range expected for the predicted ER Δ 3 protein (~63kD) [38, 39].

To identify ER Δ 3 protein we have prepared specific antibodies to this isoform. We synthesized a peptide of 22 amino acids in length homologous to the junction of exon 2/exon 4, the only unique portion of the ER Δ 3 protein. The peptide, coupled to the carrier protein ovalbumin, was injected into rabbits for polyclonal antibody production and mice in an attempt to generate monoclonal antibodies. We have recently received serum from both rabbits and mice and are in the process of testing it for specificity. In addition to making specific ER Δ 3 antibodies we have established gel conditions in order to detect ER Δ 3 protein specifically by using a combination of immunoprecipitation and western blot with an antibody specific for an epitope in the zinc finger encoded by exon 3 and one that detects an epitope in the hormone binding domain. Using these antibodies we will identify the ER Δ 3 protein *in vivo* in the event that our ER Δ 3 specific antibody is unsuccessful.

Functional Studies:

In order to understand the role ER Δ 3 in estrogen signaling, we must investigate both the distribution and function of this splice isoform. The ongoing analyses described above address the question of ER Δ 3:ER ratios *in vivo*. The subsequent data and further studies to be performed, will examine the role of ER Δ 3 in growth regulation, invasiveness and estrogen regulated gene expression.

To investigate the effects of altering the ER Δ 3:ER ratio on ER Δ 3 function, we chose ZR-75 and MCF-7 cells, both ER+, E2 responsive breast cancer cell lines. Both of these cell lines have been well-characterized with respect to responsiveness to both estrogens and antiestrogens, though they differ markedly in endogenous ER expression. MCF-7 cells contain high levels of ER, as also confirmed by our assay, and an ER Δ 3:ER mRNA ratio of approximately 1:10. In contrast, the ZR-75 cells contain low to moderate levels of ER and display ~1:5 ER Δ 3:ER ratio. The substantial amount of available information about ER actions in these cell lines allow us to define specific questions for studying effects of ER Δ 3 overexpression. Thus, we began preparing stably transfected clones of MCF-7 and ZR-75 cells overexpressing ER Δ 3.

We constructed an ER Δ 3 expression vector, by directionally ligating a restriction enzyme digested, gel purified partial ER cDNA clone, containing exon 1, 2 and 4, but missing exon 3, (gift of Miksicek et al.) into a similarly digested and purified HEGO vector containing the partial sequences of exon 1 and exons 4 through 8 of full length ER cDNA (gift of Chambon et al.). The ER Δ 3 ligation product in the parent HEGO expression vector was used to transform bacteria. DNA was purified using the Wizard Maxi-Prep kit from Promega.

Following digestion with EcoRI, the ERΔ3 coding sequence was gel purified as before, and ligated into an EcoRI digested and dephosphorylated retroviral expression vector, pMV-7, containing the neomycin resistance gene. This final mammalian expression vector with ERΔ3 under a MuLV promoter was used for all subsequent transfection experiments.

To confirm the ability of this newly constructed ERΔ3-pMV7 expression vector to express protein, the ER- Cos cells were transiently transfected and assayed for ERΔ3 expression by fluorescent immunocytochemistry with a specific antibody to the hormone binding domain of ER (H222 - gift of Greene et al.). Transient transfection was performed using lipofectin as per manufacturer's recommendations. Visualization and photography was performed using the Zeiss fluorescent microscope. Positive rhodamine staining was seen in cells transfected with ERΔ3, with no staining observed in surrounding cells. Staining indicated the ability of this construct to produce the expected protein *in vivo*. Nomarski imaging demonstrated that the specific staining was nuclear, as would be expected given that ERΔ3 retains several nuclear localization signals.

Prior to stably transfecting either MCF-7 or ZR-75 cells, sensitivity to the antiplastic geneticin (G418) for both cell lines was established. Lipofection was used to stably transfect cells with 3ug of either ERΔ3-pMV7 expression vector or an "empty" pMV7 vector control. Cells were allowed to recover overnight and placed into selection medium containing 400ug/ml (MCF-7) or 1mg/ml (ZR-75) G418, either in the absence or presence of 1×10^{-8} M Estradiol (E2). Transfected cells were maintained in these selection media for 1-2 months, at which time clones were isolated.

Since we did not observe any colonies in the ZR-75 transfections, these cells will not be discussed further. We suspect that the lack of transfection of these cells originates from their inability to form stable attachment with the substratum. From parallel transfection experiments of MCF-7 cells, we isolated approximately 25 clones of the ERΔ3-pMV7 expression vector and 20 clones of the pMV7 controls.

From the stable transfection of ERΔ3 in MCF-7 cells, yielded several G418 resistant colonies which were selected in csFBS/-E2, as well as some that were initially selected in FBS. Poor cell growth was observed by the second passage of several clones, as compared with control pMV7 transfectants. Most of the ERΔ3 transfected clones increased their growth rate by the third or fourth passage, prior to us having enough material to assay for ERΔ3 expression. However, at the fourth passage of two clones we had enough cells to plate cells in parallel into two 35mm dishes. One dish was maintained in csFBS and the other in FBS. The cells in FBS displayed significant cell death within 3 days, with one of the clones being drastically affected overnight. In contrast, the cells maintained in csFBS continued to grow. In response to this result we transferred all ERΔ3 transfected cells into csFBS containing medium. Unlike the growth inhibition by full serum observed in the ERΔ3 transfectants, pMV7 transfected controls exhibited no growth retardation in FBS supplemented serum. Subsequent RT/PCR analysis revealed low expression of ERΔ3 in several clones and all controls tested, however, one of the clones that died in the presence of FBS showed ~2:1 ratio of ERΔ3:ER mRNA compared to the 1:10 ratio present in the parental cell line. Analysis of the second clone which was more severely affected by FBS showed that at the sixth

passage no significant overexpression of ERΔ3. When the experiment of plating the cells in csFBS or FBS was repeated with these later passage cells no difference in cell growth was observed. The RT/PCR analysis identified one other ERΔ3 overexpression clone. These results suggest that the overexpression of ERΔ3 renders these cells incapable of growth in the presence of even low concentrations of estrogen (FBS contains $\sim 1 \times 10^{-12}$ M estradiol, whereas physiologic levels are $\sim 1 \times 10^{-9}$ M). It is possible that the cells that lost their sensitivity to estrogen in the later passages had undergone an adaptive mechanism to reverse the growth inhibitory effects of ERΔ3 by inhibiting its expression. Similar phenomena have been seen in studies of tumor suppressor genes.

In addition to the growth retardation, the morphology of these clones differs from the pMV7 controls and from the non-overexpressing clones. The overexpressers form circular domes in which the cells appear to be arranged around structures that approximate glandular lumens that have been observed when normal luminal mammary epithelial cells are plated on plastic. In addition the cells have are more organized and appear more cuboidal than the controls. The changes further support a less transformed phenotype resulting from ERΔ3 expression. However, though these initial observations are encouraging we must await full characterization of the stable transfectants to conclusively characterize the function of ERΔ3 in breast epithelium.

The expression of ERΔ3 protein has been confirmed in the clones that overexpressed the mRNA using the specific hormone binding domain and exon 3 specific antibodies previously described. The level of ERΔ3 protein expression is approximately equal to the amount of endogenous estrogen receptor in the MCF-7 cells (ratio of 1:1 ERΔ3:ER). The lack of gross overexpression that is often observed in transfectants further supports the growth consequences of ERΔ3 expression. Having now established both gel conditions that separate the 67kD ER from the 63kD ERΔ3 as well as *gaining* positive control cells that express ERΔ3 at a easily detectible level we will be able to proceed with the analysis of the antibodies that we have generated, as well as aid in the identification of ERΔ3 in primary breast cells.

FUTURE STUDIES

Having generated breast tumor cells stably transfected with ERΔ3, we can now perform functional studies to delineate the role of ERΔ3 in estrogen signaling. Thus MCF-7 cells overexpressing ERΔ3 will be assayed for responsiveness to estrogen and antiestrogens with endpoints such as growth, invasive potential, and estrogen target gene regulation. If ERΔ3 overexpression in fact confers a less transformed phenotype to breast cancer cells, this may suggest that of the two models presented at the end of the introduction, Model II is correct. Thus, if ERΔ3 is expressed in higher levels in normal breast epithelium than in breast tumor cells, as suggested by the normal breast cell analysis described above, a decrease or loss of ERΔ3 expression may promote cancer progression by stimulating estrogen mediated mitogenic actions. Such a mechanism of action may ultimately lead to the development of new therapies to differentiate breast tumor cells by identifying factors that regulate the estrogen receptor RNA splicing *in vivo*.

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